was electroporated into DH10B cells and titered onto a lawn of XL-1 Blue.

The murine anti-CD40 mAb variable region framework sequences were used to identify the most homologous human germline sequences. The H chain framework residues were 74% identical to human germline VH7 (7-4.1) and JH4 sequences while the L chain was 75% identical to the corresponding human germline VKIII (L6) and JK4 sequences. Alignment of the H and L chain variable sequences is shown in Fig. 1. CDR residues, as defined by Kabat et. al. (1977, 1991), are underlined and were excluded from the homology analysis. Differences in sequence are indicated by vertical lines and framework positions characterized in the combinatorial expression library are marked with an asterisk. Framework residues that differed between the murine mAb and the human templates were assessed individually.

Based on structural and sequence analysis, antibody CDRs with the exception of HCDR3 display a limited number of main chain conformations termed canonical structures (Chothia & Lesk, (1987); Chothia et. al., (1989)). Moreover, certain residues critical for determining the main chain conformation of the CDR loops have been identified (Chothia & Lesk, (1987); Chothia et. al., (1989)). Canonical framework residues of murine anti-CD40 were identified therefore, and it was determined that amino acids at all critical canonical positions within the H and L chain frameworks of the human templates were identical to the corresponding murine residues.

Surface-exposed murine amino acids not normally found in human antibodies are likely to contribute to the

immunogenicity of the humanized mAb (Padlan, (1991)). Therefore, framework residues differing between murine anti-CD40 and the human templates were analyzed and based on solvent exposure were predicted to be buried or located on the surface of the antibody (Padlan, (1991)). Solvent-exposed framework residues distal to the CDRs were not expected to contribute to antigen binding significantly and thus, with the exception of two H chain residues all were changed to the corresponding human amino acid to decrease potential immunogenicity. H chain residues 28 and 46 were predicted to be solvent exposed. However, H28 is located within the HCDR1 region as defined by Chothia & Lesk (1987) and potentially interacts with the antigen. In addition, the lysine at H46 in the murine mAb is somewhat unusual and significantly different from the glutamic acid of the human template. Therefore, the murine and human residues at H28 and H46 were expressed in the combinatorial library (Fig. 1, asterisks).

The remaining differing framework residues, all predicted to be mostly buried within the antibody, were evaluated for: (1) proximity to CDRs, (2) potential to contact the opposite domain in the V_K-V_H interface, (3) relatedness of the differing amino acids, and (4) predicted importance in modulating CDR activity as defined by Studnicka et. al., (1994). The majority of L chain framework differences in buried residues were related amino acids at positions considered not likely to be directly involved in the conformation of the CDR. However, L49 is located adjacent to LCDR2, potentially contacts the V_H domain, is unrelated to the human residue, and may be involved in determining the conformation of LCDR2. For these reasons, the murine and human amino

acids at L49 were both expressed in the combinatorial framework library (Fig. 1, asterisk).

Analysis of the murine H chain sequence and the human template was performed. Residue H9 is a proline in the murine mAb while the human template contains an unrelated serine residue. Position H9 can also play a role in modulating the conformation of the CDR and thus, was selected as a combinatorial library site (Fig. 1, asterisks). The remaining buried framework residues that differed between murine anti-CD40 and the H chain template were at framework positions 38, 39, 48, and 91. Murine anti-CD40 mAb contained glutamine and glutamic acid at H38 and H39, respectively, while the human template contained arginine and glutamine. Residue H38 is in proximity to the HCDR1, the glutamine→arginine change is non-conserved, and expression of glutamine at this site in murine Abs is somewhat unusual. Similarly, glutamic acid→glutamine is a non-conservative difference for buried amino acids, H39 is a potential V_K contact residue, and glutamic acid is somewhat unusual in murine mAbs. Residue H48 is in close proximity to HCDR2 and H91 is predicted to be a high risk site (Studnicka et. al., (1994); Harris & Bajorath, (1995)) that potentially contacts the V_K domain. Thus, both murine and human residues were expressed at H38, 39, 48, and 91 (Fig. 1, asterisks).

The combinatorial framework library (Hu I) was synthesized by the same method used to construct the chimeric anti-CD40, with modifications. Overlapping oligonucleotides encoding the framework regions of the H and L variable domains of the human template and the murine anti-CD40 CDRs as defined by Kabat et. al. (1977, 1991) were synthesized. Among these, degenerate